HIV-Specific CD4$^+$ T Cells May Contribute to Viral Persistence in HIV Controllers

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Background. Human immunodeficiency virus (HIV)–-infected individuals maintaining plasma HIV RNA levels <75 copies/mL in the absence of therapy ("HIV controllers") often maintain high HIV-specific T cell responses, which likely contribute to the control of viral replication. Despite robust immune responses, these individuals never eradicate HIV infection. We hypothesized that HIV-specific CD4$^+$ T cells might serve as target cells for HIV, contributing to viral persistence in this setting.

Methods. We measured frequencies of activated (CD38$^+$ HLA-DR$^+$) and HIV Gag-specific CD4$^+$ and CD8$^+$ T cells and plasma- and cell-associated levels of HIV RNA and DNA in a cohort of 38 HIV controllers.

Results. Although there was no evidence of a relationship between the extent of low-level viremia and the frequency of either activated or HIV-specific CD4$^+$ T cells, controllers with higher HIV-specific CD4$^+$ T cell frequencies had higher cell-associated HIV DNA levels ($\rho = 0.53$; $P = .019$). Higher activated CD4+ T cell frequencies were also associated with higher levels of cell-associated DNA ($P = .027$) and RNA ($P = .0096$). However, there was no evidence of a relationship between cell-associated HIV RNA or DNA levels and HIV-specific CD8$^+$ T cell frequencies.

Conclusions. These data support a model in which strong HIV-specific CD4$^+$ T cell responses in HIV controllers, while contributing to a potent adaptive immune response, may also contribute to viral persistence, preventing the natural eradication of HIV infection.

Intense interest has been focused on "human immunodeficiency virus (HIV) controllers," a rare group of HIV-infected individuals who maintain clinically undetectable plasma HIV RNA levels (<75 copies/mL) in the absence of antiretroviral therapy. There is now broad consensus that highly functional HIV-specific T cells play an important role in the suppression of viral replication in most of these individuals [1–13]—observations that have supported the development of T cell immunity vaccines for HIV. Because some of these individuals have maintained both clinically undetectable viremia and normal CD4$^+$ T cell counts for >2 decades, many have also hypothesized that harnessing the potent HIV-specific T cell responses observed in these individuals might allow for a "functional cure" of HIV infection. However, a recent T cell–mediated immunity vaccine for HIV failed to prevent HIV acquisition and actually increased the risk of infection in uncircumcised men [14]. There may also be negative inflammatory consequences to the immunologic control of HIV infection in HIV controllers, including systemic immune activation—occasionally contributing to significant decreases in the CD4$^+$ T cell count and to AIDS—and atherosclerosis [15, 16]. Finally, despite potent HIV-specific T cell responses and relatively small reservoirs of latently infected cells, no HIV controller has ever completely eradicated HIV [17–19].

It remains unclear why HIV controllers never succeed in completely eradicating HIV. One possibility is that the very immune response critical for the immunologic control of viral replication paradoxically promotes HIV persistence. For example, HIV-specific CD4$^+$ T cells are highly susceptible targets for direct HIV infection, because they preferentially activate and expand at sites of

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HIV replication [20]. We hypothesized that the high frequencies of activated and HIV-specific $CD4^+$ T cells observed in HIV controllers [3, 5, 6, 16] might actually serve as primary target cells, continuously replenishing the reservoir of latently infected cells and preventing complete eradication in these individuals. To begin to address this hypothesis, we assessed the relationship between activated and HIV-specific T cell responses and both plasma and cell-associated HIV RNA and DNA levels in a cohort of HIV controllers.

MATERIALS AND METHODS

Participants

HIV-infected adults were sampled from the Study of the Consequences of the Protease Inhibitor Era (SCOPE), a clinic-based cohort of >1000 HIV-infected individuals at the University of California, San Francisco. From this cohort, we sampled HIV controllers, defined as HIV-seropositive individuals maintaining clinically undetectable plasma HIV RNA levels (<75 copies/mL by bDNA or <50 copies/mL by polymerase chain reaction) for ⩾5 years in the absence of therapy, with ⩾2 plasma HIV RNA determinations in a given 1-year period. Isolated episodes of clinically detectable viremia up to 1000 copies/mL were allowed if they were followed by undetectable values. Plasma- and cell-associated levels of HIV RNA and DNA in these individuals were reported recently [19]. A subset of these individuals had cellular immunology studies performed and are the basis of the current report. All participants provided informed consent, and this research was approved by the institutional review board of the University of California, San Francisco.

Laboratory Studies

T Cell Activation Freshly collected whole-blood samples were assessed for the frequency of activated (CD38+/HLA-DR+) $CD4^+$ and $CD8^+$ T cells using 4-color flow cytometry, setting quadrant gates on isotype controls to define positive and negative populations, as described elsewhere [16]. T cell activation was assessed as the percentage of CD4+ and $CD8^+$ T cells coexpressing HLA-DR and CD38, because these measures have been associated with clinical progression in untreated individuals—even among HIV controllers—and poor $CD4^+$ T cell recovery in the presence of antiretroviral therapy [16, 21, 22].

Cytokine Flow Cytometry Fresh whole-blood samples were stimulated with overlapping peptide pools of HIV-1 p55 Gag or cytomegalovirus (CMV) pp65 protein (BD Biosciences) for 6 h, then fixed, permeabilized, and assessed for interferon (IFN)–γ and interleukin (IL)–2 expression by $CD4^+$ and $CD8^+$ T cells, as previously described (gating strategy depicted in Figure 1 of reference [9]). Cytokine secretion in an unstimulated control culture was subtracted to correct for nonspecific cytokine secretion. The frequencies of Gag-specific $CD4^+$ and $CD8^+$ T cells secreting both IFN-γ and IL-2 were used as our primary measures of the Gag-specific T cell response, because these cells are more highly enriched in HIV controllers than those producing IFN-γ alone and are believed to be more potent mediators of viral control [5, 9].

Ultra-Sensitive Plasma HIV RNA Levels Plasma HIV RNA levels were measured using an isothermal transcription mediated amplification (TMA) assay (Aptima; Gen-Probe) [23], with a 50% detection limit of 3.6–14 copies/mL when performed in singlicate [24]. The assay was performed in quadruplicate on 2 mL of plasma (obtained from the same time point as the T cell activation assays), improving the overall limit of detection to <3.5 copies/mL. The output is a ratio of signal to cutoff (S/Co; range, 0–30), with an S/Co ratio <1.0 considered undetectable [19].

Cell-Associated HIV RNA and DNA Levels The TMA assay was also used to measure cell-associated HIV RNA in cryopreserved peripheral blood mononuclear cells (PBMCs), using a modification of methods for PBMC extraction and TMA amplification of hepatitis C virus [25]. S/Co ratios were normalized to the input number of PBMCs. Total proviral HIV DNA was extracted from PBMCs using published methods and normalized to the input number of PBMCs [26]. This assay has an overall sensitivity of 1 copy/3 μg of input DNA (~450,000 PBMCs) [27].

Statistical Methods

Relationships between continuous variables were assessed with Spearman rank order correlation coefficients. Adjusted associations were assessed with linear regression, including potential confounders associated with the outcome of interest in unadjusted models ($P > .10$) and calculating standard errors with heteroskedasticity-consistent covariance matrix estimators [28]. Models were considered valid only if inferences were robust to exclusion of influential outliers and leverage points.

RESULTS

Characteristics of Participants

A total of 38 HIV controllers with plasma HIV RNA levels <75 copies/mL in the absence of antiretroviral therapy and available cellular immunology data contributed to these analyses. Most were 40–50 years of age, and nearly one-half were women (Table 1). Most had received a diagnosis of HIV infection >1 decade previously, and most had normal $CD4^+$ T cell counts (median, 746 cells/mm^3; interquartile range, 564-1037 cells/mm^3). Only 3 HIV controllers (7%) had a CD4+ T cell count <350 cells/mm^3. Fourteen (37%) had at least 1 HLA B*57 allele.

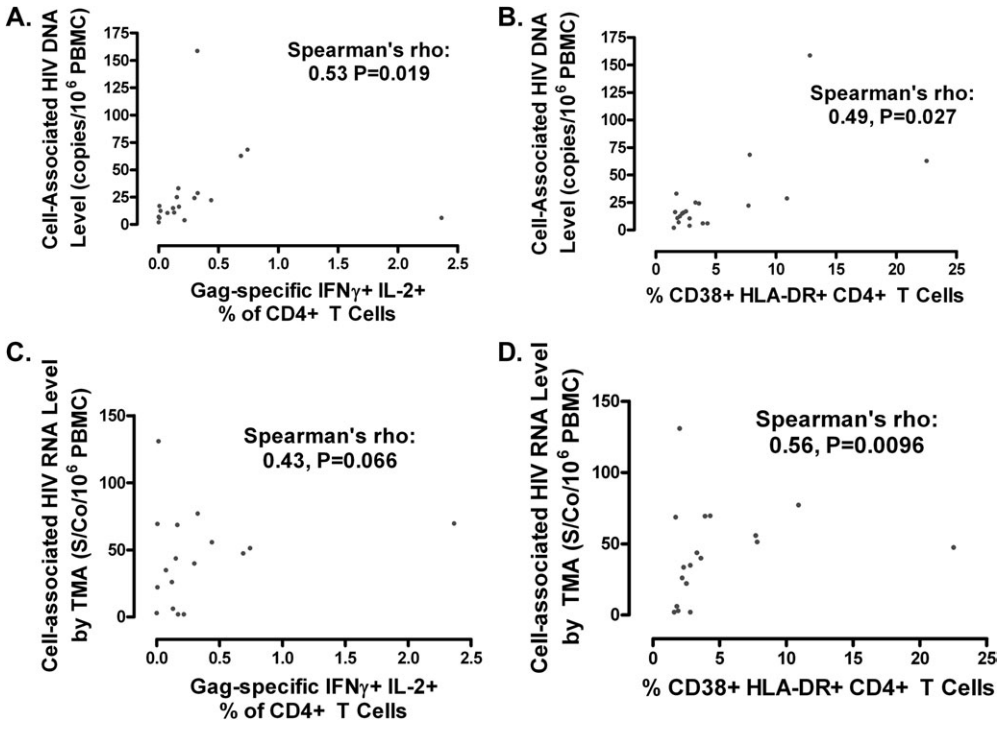

Figure 1. Strong relationship between cell-associated human immunodeficiency virus (HIV) burden and the frequency of activated and HIV-specific $CD4^+$ T cells in HIV controllers. The association between cell-associated HIV DNA and RNA levels and the frequencies of activated ($CD38^+$ $HLA\text{-}DR^+$) and HIV Gag-specific $CD4^+$ T cells was assessed in peripheral blood mononuclear cells (PBMCs) from 19 HIV controllers. Higher cell-associated HIV DNA levels were strongly associated with higher frequencies of Gag-specific $CD4^+$ T cells *(A)* and activated $CD4^+$ T cells *(B)*. Similarly, higher cell-associated HIV RNA levels were associated with higher frequencies of activated $CD4^+$ T cells *(D)* and tended to be associated with higher frequencies of Gag-specific $CD4^+$ T cells *(C)* .IFN, interferon; IL, interleukin; S/Co, ratio of signal to cutoff; TMA, transcription mediated amplification.

Weak and Inconsistent Relationships between the Extent of Low-Level Viremia and the Frequency of Activated and HIV-Specific T Cells

The extent of low-level viremia was assessed in all 38 HIV controllers using a high-sensitivity TMA assay. As previously reported, although all HIV controllers had undetectable plasma HIV RNA levels, as determined using clinically available assays, most HIV controllers had detectable HIV RNA in plasma by TMA (median S/Co ratio, 7.1; interquartile range, 1.8–18.8) [19]. Because the extent of low-level viremia might be a major determinant of the frequency of activated and HIV-specific T cells in these individuals, we assessed the relationship between these measurements. Although higher plasma HIV RNA levels were modestly correlated with higher frequencies of activated ($CD38^+$ $HLA\text{-}DR^+$) $CD8^+$ T cells ($\rho = 0.32$; $P = .048$), there was no evidence for a relationship between the extent of low-level viremia and the frequencies of activated $CD4^+$ T cells ($\rho = 0.12$; $P = .48$), HIV Gag-specific IFNγ+IL-2+ $CD4^+$ T cells ($\rho = -0.1$; $P = .73$), or HIV Gag-specific IFNγ+IL-2+ CD8+ T cells ($\rho = -0.04$; $P = .81$).

Strong Relationship between Activated and HIV-Specific $CD4^+$ T Frequencies and Cell-Associated HIV Burden

Although the extent of low-level viremia did not appear to be a major determinant of the frequency of activated and HIV-specific $CD4^+$ T cells, we hypothesized that the HIV-specific and activated

Table 1. Characteristics of Human Immunodeficiency Virus (HIV) Controllers

Characteristic	Data ($n = 38$)
Age, median years (IQR)	47 (44–50)
No. (%) of female subjects	16 (42)
CD4 cell count, median cells/mm^3 (IQR)	746 (564–1037)
Plasma HIV RNA level (branched DNA assay), median $\log_{10}$ copies/mL	<75
Years of diagnosis of HIV infection	14 (8–18)
No. (%) of subjects with HLA $B57^+$	14 (37)

$CD4^+$ T cell expansion observed in most HIV controllers might increase the availability of target cells for HIV infection, contributing to viral persistence. To address this, we assessed the relationship between the activated and HIV-specific $CD4^+$ T cell frequencies and cell-associated reservoirs of HIV RNA and DNA in PBMCs in a subset of 18 HIV controllers. As expected, higher cell-associated HIV DNA levels were strongly associated with higher frequencies of HIV Gag-specific IFN-γ+IL-2+ $CD4^+$ T cells ($\rho = 0.53$; $P = .019$, Figure 1A) and activated $CD4^+$ T cells ($\rho = 0.49$; $P = .027$) (Figure 1B). Similarly, higher cell-associated HIV RNA levels were associated with higher frequencies of activated $CD4^+$ T cells ($\rho = 0.56$; $P = .0096$) (Figure 1D) and tended to be associated with higher frequencies of Gag-specific IFN-γ+IL-2+ $CD4^+$ T cells ($\rho = 0.43$; $P = .066$) (Figure 1C). Similar trends were observed when the Gag-specific IFN-γ+ (with or without IL-2) $CD4^+$ T cell frequency was used to quantify HIV-specific $CD4^+$ T cells and when HLA-DR+ (with or without CD38) $CD4^+$ T cells was used to assess activated $CD4^+$ T cells. Confirming that the relationship between HIV-specific CD4+ T cells and cellular HIV burden was antigen specific, there was no evidence for an association between cell-associated HIV RNA or DNA levels and CMV pp65-specific IFN-γ+IL-2+ $CD4^+$ T cell frequencies ($P > .27$ for both).

We also assessed for potential confounders of these relationships. Although there was no evidence of an association between age, sex, CD4 cell count, or HLA B*57 status and cell-associated HIV DNA or RNA levels (all $P > .10$), longer duration of HIV diagnosis (a variable underestimate of the duration of HIV infection) was associated with higher cell-associated HIV DNA levels ($\rho = 0.47$; $P = .048$). However, there was no evidence of a relationship between time since HIV diagnosis and activated or HIV-specific $CD4^+$ T cell frequencies ($P > .59$ for both). Furthermore, after adjustment for time since HIV diagnosis, the activated and HIV-specific $CD4^+$ T cell frequencies continued to be strongly associated with cell-associated DNA levels ($P < .001$ for both). It should be noted, however, that 2 influential outliers (one with the highest HIV DNA level and one with the highest HIV-specific $CD4^+$ T cell frequency in Figure 1A) needed to be excluded from the HIV-specific $CD4^+$ T cell model, because they acted as disproportionally influential leverage points that prevented modeling a linear relationship.

No Evidence of a Relationship between Activated and HIV-Specific CD8+ T Cell Frequencies and Cell-Associated HIV Burden

Because higher cellular burden of HIV could potentially be a cause rather than a consequence of activated and HIV-specific $CD4^+$ T cell expansion, we also assessed the relationship between cell-associated HIV burden and the frequency of activated and HIV-specific $CD8^+$ T cell responses. If a higher cell-associated burden of HIV was causing greater expansion of activated and HIV-specific $CD4^+$ T cells, we would also expect to see a strong relationship between cellular HIV burden and activated and HIV-specific $CD8^+$ T cells. However, cell-associated HIV burden was less consistently associated with HIV-specific and activated $CD8^+$ T cells. Although cell-associated HIV DNA levels were associated with activated $CD8^+$ T cells ($\rho = 0.46$; $P = .041$) (Figure 2B), there was no evidence of an association with HIV-specific IFN-γ+IL-2+ $CD8^+$ T cells ($\rho = -0.18$, $P = .47$, Figure 2A). There was also no evidence for an association between cell-associated HIV RNA levels and frequencies of either HIV-specific IFN-γ+IL-2+ $CD8^+$ T cells ($\rho = -0.33$; $P = .17$) (Figure 2C) or activated $CD8^+$ T cells ($\rho = 0\ .38$; $P = .10$) (Figure 2D). There was also no evidence of an association between the frequency of Gag-specific IFN-γ+ (with or without IL-2) $CD8^+$ T cells and either cell-associated HIV RNA or DNA levels ($P > .45$ for both).

DISCUSSION

Although most HIV controllers maintain strong HIV-specific T cell responses and extremely low levels of virus replication in the absence of therapy, no controller has ever eradicated HIV. We hypothesized that HIV-specific $CD4^+$ T cells, although potentially supporting an effective HIV-specific immune response, might also serve as major target cells for HIV, continually replenishing the reservoir of latently infected cells. Supporting this hypothesis, we found that HIV controllers with the highest frequencies of activated and HIV-specific $CD4^+$ T cells had the highest cell-associated burdens of HIV. Although higher viral burden could also be a cause of greater activated and HIV-specific $CD4^+$ T cell expansion, we observed no evidence of a relationship between the degree of low-level viremia and the frequency of these cells. Furthermore, we found no evidence of a relationship between the cell-associated viral burden and HIV-specific $CD8^+$ T cell frequencies. Collectively, these data support a model in which HIV-specific $CD4^+$ T cells may replenish the latent reservoir and prevent the natural eradication of HIV infection in HIV controllers.

Our results are consistent with a recent report demonstrating that $CD4^+$ T cells from HIV controllers are just as easily infected with HIV after ex vivo stimulation as cells from HIV-uninfected individuals [29]. Although viruses from HIV controllers may be somewhat less fit than viruses from viremic individuals [30, 31], replication-competent virus can be isolated from many controllers and some have been infected with highly pathogenic viruses from individuals with AIDS [32–34]. Despite infection with replication-competent viruses and having susceptible $CD4^+$ T cells, HIV controllers maintain much lower cell-associated DNA levels than do HIV-infected individuals maintaining treatment-mediated viral suppression [17–19]. These exceptionally small cellular reservoirs in HIV controllers have been

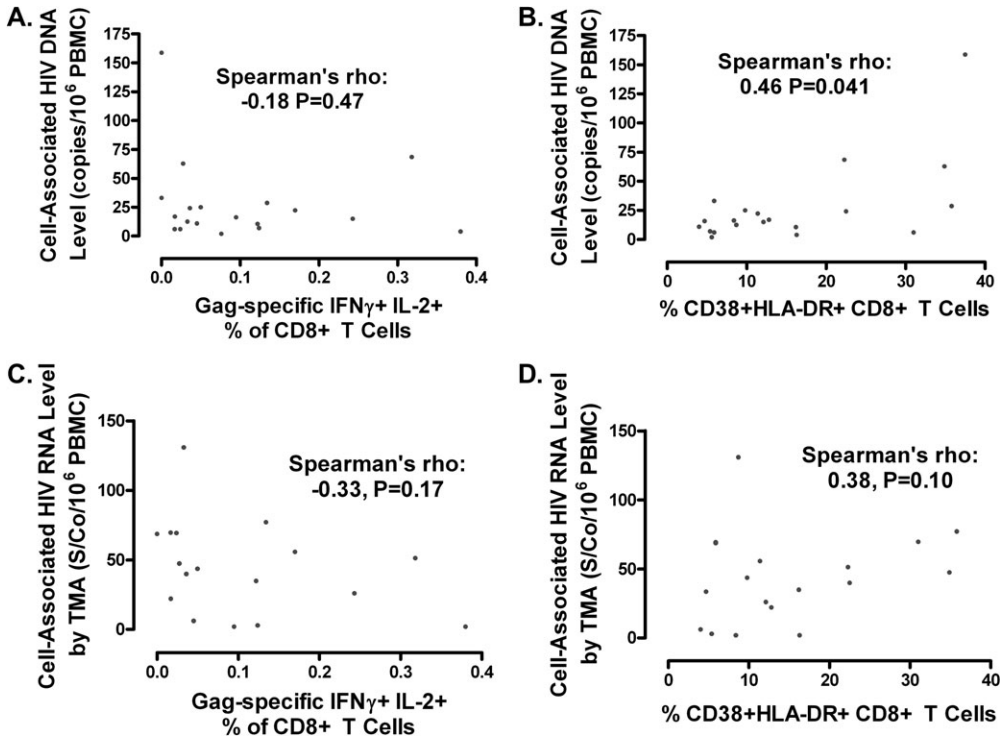

Figure 2. Weak and inconsistent relationships between cell-associated human immunodeficiency virus (HIV) burden and the frequency of activated and HIV-specific $CD8^+$ T cells in HIV controllers. The association between cell-associated HIV DNA and RNA levels and the frequencies of activated and HIV Gag-specific $CD8^+$ T cells was assessed in peripheral blood mononuclear cells (PBMCs) from 19 HIV controllers. Although higher frequencies of activated $CD8^+$ T cells were associated with higher cell-associated HIV DNA levels *(B),* there was no evidence of a relationship between the frequency of HIV Gag-specific CD8+ T cells and cell-associated HIV DNA levels *(A).* There was also no evidence for a relationship between either HIV Gag-specific *(C)* or activated *(D)* $CD8^+$ T cell frequencies and cell-associated HIV RNA levels. IFN, interferon; IL, interleukin; S/Co, ratio of signal to cutoff; TMA, transcription mediated amplification.

largely attributed to a highly potent cytotoxic HIV-specific $CD8^+$ T cell response, observable in the majority of these individuals [1–13]. Enrichment for certain HLA and KIR allotypes in HIV controllers has also implicated a potential role of natural killer cells in clearing virally infected cells [35]. Despite these potent cytotoxic antiviral responses, HIV controllers never completely eradicate HIV. Our results are consistent with the hypothesis that the very HIV-specific $CD4^+$ T cell response coordinating an effective antiviral immune response may be contributing to viral persistence in this setting.

It has long been known that HIV-specific $CD4^+$ T cells are more likely to be infected with HIV than non–HIV-specific $CD4^+$ T cells [20]. It is worth carefully considering this point in the context of HIV controllers because—as a group—they harbor extremely high frequencies of HIV-specific $CD4^+$ T cells while maintaining extremely low proviral HIV DNA levels [3, 5, 6,17–19]. Taken together, these observations might suggest that the latent reservoir in HIV controllers is more highly concentrated in the HIV-specific $CD4^+$ T cell population than in most other HIV-infected individuals. This might be the case if HIV controllers were primarily containing viral replication in lymphoid tissues with high local concentrations of expanded HIV-specific T cells. If this hypothesis were true, it might have important implications for eradication strategies. For example, if reinfection of HIV-specific $CD4^+$ T cells could be prevented in HIV controllers with combination antiretroviral therapy—preferably composed of agents targeting preintegration steps—repeated therapeutic vaccination with HIV antigens could potentially stimulate virus production by latently infected HIV-specific $CD4^+$ T cells, allowing their clearance by the potent antiviral responses maintained by most of these individuals, potentially reducing the latent reservoir to low-enough levels that eradication might be possible. Although such a strategy would not have immediate relevance to the majority of HIV-infected individuals who fail to spontaneously control HIV replication, it might provide proof of principle that harnessing host anti-viral immune responses could play a role in HIV eradication.

The recognition that HIV-specific $CD4^+$ T cells are likely to be major target cells for HIV even in controllers may also have relevance for T cell immunity vaccine strategies for HIV. Vaccines that elicit strong proliferative HIV-specific $CD4^+$ T cell

responses might actually increase the risk of HIV acquisition, as appeared to be the case in the recent STEP trial [14]. Similarly, a therapeutic vaccine inducing a strong CD4+ T cell response actually shortened the time to viral rebound among HIV-infected individuals undergoing treatment interruption [36], though this latter observation has not been observed with all therapeutic HIV vaccines [37]. Indeed, low systemic immune activation levels have been consistently associated with a decreased risk of HIV acquisition in highly exposed HIV-uninfected individuals [38–42], and blocking the recruitment of activated $CD4^+$ T cell targets to mucosal surfaces with a topical microbicide appeared to protect macaques from systemic infection by the simian immunodeficiency virus following vaginal challenge [43].

Our study has several limitations that deserve comment. First, our study is correlative, and we do not provide definitive proof that activated and HIV-specific $CD4^+$ T cells are major contributors to viral persistence in HIV controllers. Indeed, higher cell-associated viral burden could be a cause rather than a consequence of the expansion of activated and HIV-specific $CD4^+$ T cells observed. However, we found no evidence of an association between the extent of low-level viremia and the expansion of these cells. Furthermore, if higher viral burden was a major determinant of activated and HIV-specific T cell expansion in HIV controllers, we might have also expected to see strong and consistent associations between cell-associated viral burden and the frequency of HIV-specific $CD8^+$ T cells. However, we saw, at best, inconsistent evidence of these relationships. The lack of consistent relationships between cell-associated viral burden and activated and HIV-specific $CD8^+$ T cell responses might be explained by the countervailing antiviral activity of these cells. However, the contrasting relationships between cell-associated viral burden and HIV-specific $CD4^+$ and $CD8^+$ T cells further highlights the relative failure of HIV-specific $CD4^+$ T cells to prevent viral persistence. Ultimately, isolating $CD4^+$ T cells from blood and tissues and measuring HIV DNA levels separately within HIV-specific and non–HIV-specific fractions would be the most definitive way to assess whether the cellular viral burden in HIV controllers is preferentially maintained in the HIV-specific population [20], but this is beyond the scope of the current study. Finally, not every HIV controller with high HIV-specific CD4+ T cell frequencies in our sample had high HIV DNA levels. In fact, the controller with the highest HIV-specific $CD4^+$ T cell frequency had a very low HIV DNA level. Additional testing is necessary to address whether this individual has favorable CCR5 genetics or other important cellular restriction factors.

In summary, although most HIV controllers maintain high frequencies of potent HIV-specific T cells, none has ever successfully eradicated HIV. The strong relationship between the frequency of activated and HIV-specific $CD4^+$ T cells and cell-associated viral burden in controllers may suggest that these cells are continually replenishing the latent reservoir, preventing the natural eradication of HIV in this setting. These observations have important implications both for the design of effective HIV vaccines and microbicides and for novel HIV eradication strategies.

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